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Heterologous amplification of homologous beta-adrenoceptor desensitization in airway smooth muscle

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**Protein kinase C amplifies
catecholamine inactivation by
catechol *O*-methyltransferase in
airway smooth muscle.
Implications for asthma.**

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Abstract

Methacholine-induced activation of protein kinase C (PKC) has been found to reduce the functional antagonism of bovine tracheal smooth muscle contraction by β -adrenoceptor agonists. In addition, catechol-*o*-methyltransferase (COMT) is able to inactivate endogenous and exogenous catecholamines. By reducing the β -adrenergic response, increased PKC and COMT activities could play an important role in asthma. In this study, we examined the effects of a COMT-inhibitor (OR 486) and a PKC inhibitor (GF 109203X) on isoprenaline- and fenoterol-induced relaxation of bovine tracheal smooth muscle. In the presence of OR 486 (10 μ M), the isoprenaline concentration-relaxation-curve from 1 μ M methacholine-induced smooth muscle tone was shifted to the left by over one log-unit (pD_2 of 7.58 ± 0.15 vs 6.37 ± 0.14 in controls, $P < 0.001$). Maximum relaxation (E_{max}) remained unchanged, indicating that COMT only decreases the potency of isoprenaline. Very similar results were found using the classical COMT-inhibitor tropolone. GF 109203X (10 μ M) induced a leftward-shift of the relaxation curve of isoprenaline of very similar magnitude as OR 486 and tropolone (pD_2 of 7.46 ± 0.13 vs 6.37 ± 0.14 in controls, $P < 0.001$); however E_{max} was also significantly increased (92.3 ± 1.8 vs $82.8 \pm 3.3\%$ in controls, $P < 0.01$). In the combined presence of OR 486 and GF 109203X no additive effects were found on the pD_2 value of isoprenaline (7.56 ± 0.13), indicating a common pathway for PKC and COMT in attenuating the β -adrenoceptor responsiveness towards the β -agonist, presumably through activation of COMT by PKC. As expected, OR 486 did not affect relaxation by the β -agonist fenoterol, which lacks the catechol moiety. GF 109203X, however, significantly enhanced E_{max} (78.7 ± 3.9 vs 58.4 ± 7.6 in controls, $P < 0.05$), while the potency of fenoterol remained unchanged. For both isoprenaline and fenoterol, similar results were found with the COMT and PKC inhibitors and their combination when 10 μ M, instead of 1 μ M, methacholine was used to induce maximal smooth muscle contraction. The results indicate that, in bovine tracheal smooth muscle preparations, activation of COMT by PKC reduces functional antagonism of methacholine-induced contraction by catecholamines. Both PKC and COMT, as well as the interaction between the two, may therefore play a substantial role in attenuating an important, endogenous β -adrenoceptor-mediated, defence in patients with (severe) asthma.

Introduction

It is widely accepted that the efficacy of endogenous and exogenous β -adrenoceptor agonists in asthma is mainly due to functional antagonism counteracting the bronchoconstrictor effects of neurotransmitters and mediators released in airway inflammation [1]. In patients with asthma, circulating adrenaline is importantly involved in controlling airway smooth muscle tone [2], as indicated by the detrimental effects of β -adrenoceptor antagonists, including bronchospasm and aggravation of the disease, as well as increased bronchial responsiveness to methacholine and histamine [3-8]. In contrast, in non-asthmatics even high doses of β -blockers have little or no effect on airway function

and -responsiveness [9,10]. Inhaled β_2 -adrenoceptor agonists are first choice in the acute relief of asthma symptoms [11]. However, a diminished effectiveness of β -adrenoceptor agonists is observed during a severe exacerbation of asthma [12]. This is presumably due to mediator- and/or neurotransmitter- induced activation of protein kinase C (PKC) and subsequent uncoupling of the β -adrenoceptor from the effector system, as indicated by our recent observation in bovine tracheal smooth muscle that contractile agonist-induced PKC-activation importantly reduced the relaxant responsiveness towards isoprenaline [13]. However, in additional experiments we observed a marked difference in the involvement of PKC in modulating isoprenaline- and fenoterol-induced relaxation. Thus, in bovine tracheal smooth muscle preparations contracted with methacholine, inhibition of PKC enhanced both potency (pD_2) and efficacy (E_{max}) of isoprenaline [13], whereas with fenoterol the potency but not the efficacy was increased after PKC-inhibition (Boterman et al., unpublished observations). Isoprenaline, like adrenaline being a catecholamine, is a substrate for catechol-*O*-methyltransferase (COMT), which transfers a methyl group from 5-adenylyl methionine to the 3-OH substituent. The conjugated compound has no adrenergic activity and may be either excreted or further metabolized by monoamine oxidase [14]. High COMT activities have been described in human and rat lung [15,16] and COMT has proved functionally operative in airway smooth muscle of various animals. Thus, *O*-methylation of both 3H-noradrenaline and 3H-isoprenaline [16-18] as well as increased potency of isoprenaline-induced relaxation after inhibition of COMT has been reported [19,20].

Since, fenoterol, a resorcinolamine, is not a substrate for COMT, it is tempting to speculate that COMT could account for the differences between isoprenaline and fenoterol mentioned above. In addition, by reducing the β -adrenergic response, both PKC and COMT, as well as a possible interaction between the two enzymes, may play an important role in decreasing the effectiveness of circulating adrenaline, a major endogenous β -adrenoceptor mediated defence in severe asthma. In this study we found functional evidence for the involvement of COMT, and its activation by PKC, in isoprenaline-induced relaxation of methacholine-contracted bovine tracheal smooth muscle, by using a specific, nonselective PKC inhibitor GF 109203X [21] and two potent and specific COMT-inhibitors, OR 486 [22] and tropolone [23,24].

Materials and Methods

Tissue preparation

Fresh bovine tracheas were obtained from the slaughterhouse and were transported to the laboratory within 30 min at room temperature in Krebs-Henseleit (KH) buffer of the following composition (nM): NaCl 177.5, KCl 5.6, $MgSO_4$ 1.2, $CaCl_2$ 2.5, NaH_2PO_4 1.3, $NaHCO_3$ 25.0, glucose 5.5, pregassed with 95% O_2 and 5% CO_2 ; pH 7.4. The tracheal smooth muscle was dissected carefully and smooth muscle strips (12x3 mm) were prepared free of mucosa and serosal connective tissue in KH buffer gassed with 95% O_2 /5% CO_2 at

room temperature. Subsequently, all strips were maintained overnight in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10 mM NaHCO₃, 20 mM HEPES, 100 U/ml penicillin, 100 µg/ml streptomycin and 10% fetal calf serum at 37°C (55 rpm).

Experimental procedure

After washing in several volumes of KH-buffer, gassed with 95% O₂ and 5% CO₂, pH 7.4 at 37°C, the bovine tracheal smooth muscle preparations were mounted in 20 ml organ baths containing gassed KH-buffer (37°C) for isotonic recording, using a preload of 500 mg. No basal myogenic tone was observed in bovine tracheal smooth muscle. After a 60 min equilibration period the strips were precontracted twice with methacholine (0.1, 1, 10 and 0.1, 1, 10, 100 µM, respectively) with a 60 min washing period in between. Maximal relaxation was established with isoprenaline (0.1 µM), immediately followed by a 30 min washing period.

Cumulative concentration response curves were made with methacholine (1 nM – 100 µM) in the absence and presence of 1 µM OR 486 and/or 10 µM GF 109203X. In separate experiments it was found that the concentration of GF 109203X caused complete inhibition of 10 µM phorbol 12-myristate 13-acetate (PMA)-induced contraction. In addition, in bovine tracheal [13] and rat oesophagus [25] smooth muscle it has been demonstrated that PKA-dependent, but β-adrenoceptor independent relaxation, induced by either forskolin or IMBX, is not affected by GF 109203X, indicating no nonspecific effects of the PKC-inhibitor in both airway and oesophagus smooth muscle relaxation.

For relaxation studies, the preparations were preincubated with KH-buffer (45 min), 1 µM OR 486 (30 min) or 100 µM tropolone (30 min) or 10 µM GF 109203X (45 min) or the combination of 1 µM OR 486 and 10 µM GF 109203X. Subsequently, smooth muscle tone was raised with methacholine at 1 or 10 µM, which was gradually built up in 3 or 4 concentration steps, respectively. Cumulative concentration-relaxation-curves (CRCs) were constructed using (-)-isoprenaline (0.1 nM – 100 µM) or fenoterol (0.1 nM – 10 µM), added in 0.5 log increments. At the end of each experiment the smooth muscle strips were washed twice and maximal relaxation was re-established with 10 and 100 µM isoprenaline. In all experiments, the slight reduction of methacholine-induced smooth muscle tone, as a consequence of the preincubation condition, was carefully compensated for by additional administration of small amounts of the contractile agonist, before relaxation curves were obtained.

Data Analysis

Responses were expressed as percentages of the response to 100 µM methacholine in the second precontraction in each experiment, with reference to basal tone as established at the end of each experiment. All data are presented as mean ± S.E.M. Curves were fitted using the logistic 4-parameter model (Sigmaplot 9.0). Statistical analysis was performed by means of the two-tailed Student's *t*-test for paired or unpaired observations. *P* values < 0.05 were considered statistically significant.

Materials

Dulbecco's modification of Eagle's Medium (DMEM), foetal bovine serum, NaHCO_3 solution (7.5%), penicillin/streptomycin solution (5000 U/ml ; 5000 $\mu\text{g/ml}$) and HEPES solution (1 M) were obtained from Gibco BRL Life Technologies. (Paisley, U.K.). Methacholine chloride and (-)-isoprenaline hydrochloride were obtained from Sigma Chemical Co. (St. Louis, MO, U.S.A.), GF 109203X (2-[1-(3 imethylaminopropyl)-1H-indol-3-yl]-3-(1H-indol-3-yl) maleimide) was purchased from RBI (Natick, MA, USA) and fenoterol hydrobromide was a generous gift from Boehringer-Ingelheim (Ingelheim, Germany). OR 486 was obtained from Tocris Bioscience (Avonmouth, UK) and tropolone was a gift from Prof. B.H.C. Westerink (Groningen University, The Netherlands). All other chemicals were of analytical grade.

Results

Effect of OR 486 and GF 109203X on methacholine-induced contraction

GF 109202X caused a small shift to the right of the cumulative concentration-contraction curve of methacholine in bovine tracheal smooth muscle, with a reduction in pD_2 ($-\log \text{EC}_{50}$) value from 7.01 ± 0.01 to 6.58 ± 0.04 , while the maximal contractile effect (E_{max}) was unchanged (Fig. 1). For OR 486 no effect was observed on the methacholine-induced contraction ($\text{pD}_2 = 6.99 \pm 0.04$). In addition, OR 486 had no further effect on the small rightward shift of methacholine-induced contraction in the presence of GF 109203X (Fig.1).

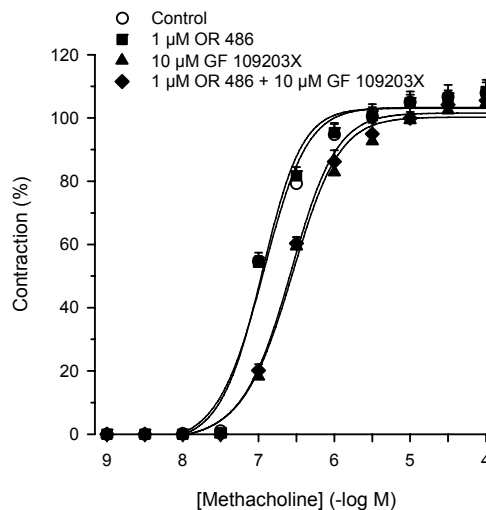


Figure 1 Cumulative concentration-response curves of methacholine-induced bovine tracheal smooth muscle contraction in the absence and presence of 10 μM GF 109203X, 1 μM OR 486 and the combination of 10 μM GF 109203X and 1 μM OR 486. Results are obtained from 2 experiments performed in duplicate.

Effect of OR 486 and tropolone on isoprenaline- induced relaxation

To determine the effect of COMT on isoprenaline-induced relaxation, we used two different COMT inhibitors. In the presence of the potent COMT inhibitor OR 486 (1 μ M), the pD_2 but not E_{max} values of isoprenaline-induced relaxations were significantly enhanced at the two methacholine-induced contraction levels applied (Fig. 2A), pD_2 values increasing from 6.15 ± 0.08 to 7.44 ± 0.16 ($P < 0.05$) for 10 μ M methacholine-induced contraction and from 6.95 ± 0.09 to 7.96 ± 0.14 ($P < 0.01$) for 1 μ M methacholine-induced contraction. Similarly, in the presence of the classical COMT inhibitor tropolone (100 μ M), similar increases in the isoprenaline pD_2 values were found (Fig. 2B), the left shifts being from 6.24 ± 0.01 to 7.39 ± 0.06 ($P < 0.01$) and from 6.91 ± 0.09 to 7.77 ± 0.01 ($P < 0.05$) for 10 and 1 μ M methacholine-induced contraction, respectively. Again, E_{max} values were not affected, indicating that inhibition of COMT only enhanced the potency of isoprenaline, not the efficacy.

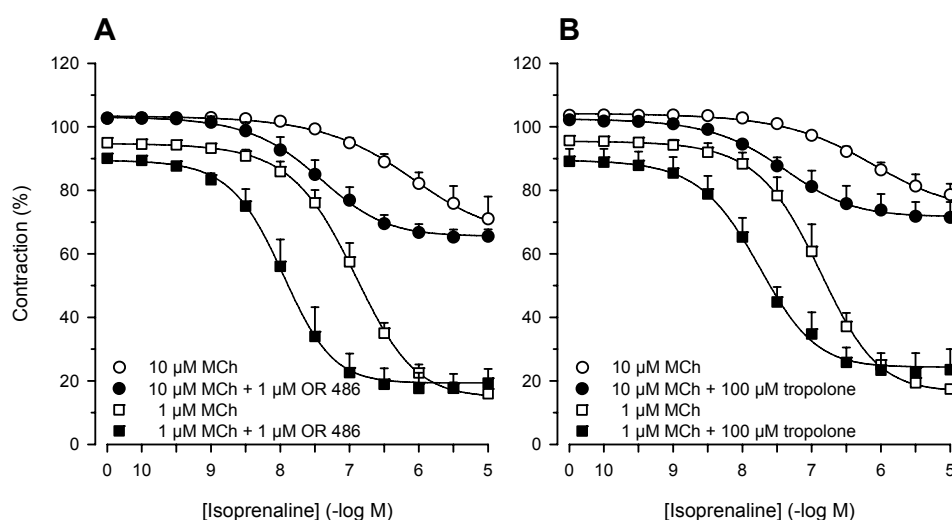


Figure 2 (-)-Isoprenaline-induced relaxation of bovine tracheal smooth muscle preparations precontracted with 1 and 10 μ M methacholine (MCh) in the absence and presence of 1 μ M OR 486 (Panel A) and 100 μ M tropolone (Panel B). Results are means \pm S.E.M. of 3 experiments each performed in duplicate.

Effect of OR 486 and GF 109203X on isoprenaline-induced relaxation

In the presence of GF 109203X (10 μ M) isoprenaline-induced relaxation from a 10 μ M methacholine-induced smooth muscle tone, is also significantly potentiated, to a similar extent as with OR 486, as no significant difference was found between the two pD_2 values (Fig. 3A; Table 1). Interestingly, and in contrast to the inhibition of COMT, PKC-inhibition

also increased E_{\max} of isoprenaline, indicating that, in addition to potency, PKC is also reducing the efficacy of the β -agonist. Furthermore, in the presence of both OR 486 and GF 109203X no additional effects were found on pD_2 and E_{\max} values of isoprenaline (Table 1), compared to treatment with GF 109203X alone.

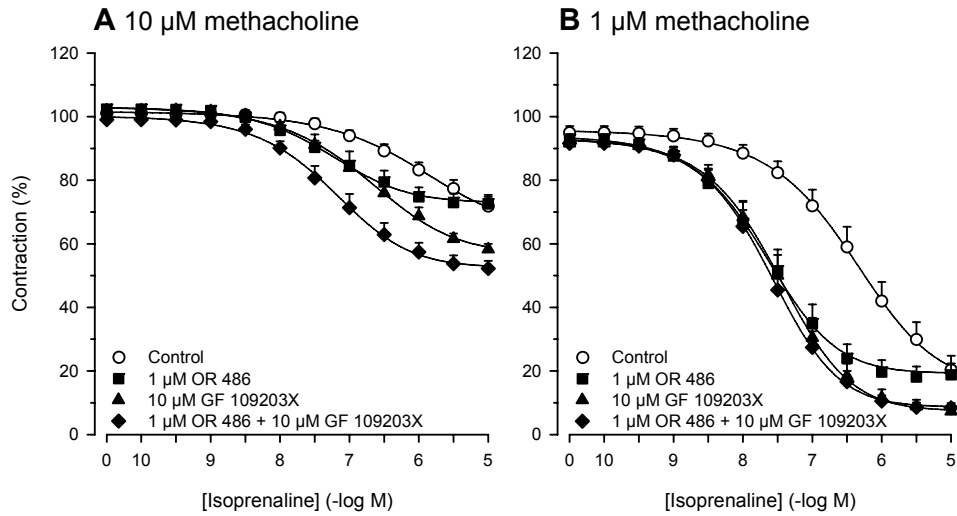


Figure 3 (-)-Isoprenaline-induced relaxation of bovine tracheal smooth muscle preparations precontracted with 10 (Panel A) and 1 (Panel B) μ M methacholine in the absence and presence of 10 μ M GF 109203X, 1 μ M OR 486 or the combination of 10 μ M GF 109203X and 1 μ M OR 486. Results are means \pm S.E.M. of 4-6 experiments each performed in duplicate.

Table 1 Maximal effect (E_{\max}) and potency (pD_2) of (-)-isoprenaline-induced relaxation of bovine tracheal smooth muscle contracted by 1 or 10 μ M methacholine (MCh), in the absence and presence of 10 μ M GF 109203X, 1 μ M OR 486 and the combination of 10 μ M GF 109203X and 1 μ M OR 486.

Preincubation	1 μ M MCh		10 μ M MCh	
	E_{\max} (%)	pD_2 (-log M)	E_{\max} (%)	pD_2 (-log M)
Control	82.8 \pm 3.3	6.37 \pm 0.14	34.9 \pm 3.5	5.96 \pm 0.13
GF 109203X	93.0 \pm 1.8***	7.46 \pm 0.13***	45.4 \pm 2.9*	6.63 \pm 0.19*
OR 486	81.6 \pm 3.2	7.58 \pm 0.15***	29.6 \pm 2.9	7.20 \pm 0.24***
GF 109203X + OR 486	91.2 \pm 1.6***	7.56 \pm 0.13***	48.6 \pm 2.4**	7.09 \pm 0.20***

Data represent means \pm S.E.M. of 4-6 experiments, each performed in duplicate. Significantly different from control: * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$.

At the lower submaximal contraction level induced by 1 μ M methacholine, at which the isoprenaline-induced relaxation is much more pronounced, again, the potency of

isoprenaline is significantly enhanced in the presence of OR 486, with no effect on maximal relaxation (Table 1). GF 109203X induced a marked leftward shift of the isoprenaline-induced relaxation, similar to that by OR 486, and significantly increased maximal relaxation of isoprenaline as well. Again, combined treatment with OR 486 and GF 109203X had no additional effects on pD_2 and E_{max} values of isoprenaline (Table 1), compared to GF 109203X alone.

Effect of OR 486 and GF 109203X on fenoterol-induced relaxation

We used fenoterol to dissect the effects of COMT- and PKC-inhibition on the relaxation induced by a β_2 -adrenoceptor agonist because fenoterol is lacking the catechol moiety. As expected, fenoterol-induced relaxation from both the 1 and 10 μ M methacholine-induced contraction level was not affected by OR 486 (Fig. 4A, B; Table 2). Remarkably, for both 1 and 10 μ M methacholine-induced contractions, maximal relaxation of fenoterol was significantly enhanced in the presence of GF 109203X, with no effect on the potency (Fig. 4A, B; Table 2). This is in striking contrast with isoprenaline-induced relaxation, where inhibition of PKC also markedly enhanced the potency of isoprenaline. Again, in accordance with the results described above, the combined treatment with OR 486 and GF 109203X had no additional effects on fenoterol-induced relaxation for both contraction levels, compared to GF 109203X alone (Table 2).

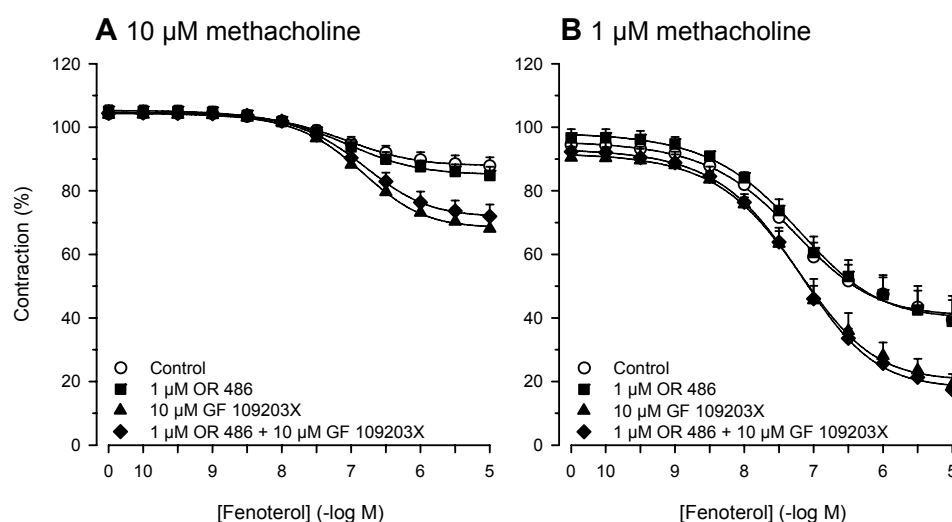


Figure 4 Fenoterol-induced relaxation of bovine tracheal smooth muscle preparations precontracted with 10 (Panel A) and 1 (Panel B) μ M methacholine in the absence and presence of 10 μ M GF 109203X, 1 μ M OR 486 or the combination of 10 μ M GF 109203X and 1 μ M OR 486. Results are means \pm S.E.M. of 4 experiments each performed in duplicate.

Table 2 Maximal effect (E_{max}) and potency (pD_2) of fenoterol-induced relaxation of bovine tracheal smooth muscle contracted by 1 or 10 μ M methacholine (MCh), in the absence and presence of 10 μ M GF 109203X, 1 μ M OR 486 and the combination of 10 μ M GF 109203X and 1 μ M OR 486.

Preincubation	1 μ M MCh		10 μ M MCh	
	E_{max} (%)	pD_2 (-log M)	E_{max} (%)	pD_2 (-log M)
Control	58.4 \pm 7.6	7.34 \pm 0.11	15.7 \pm 2.5	7.09 \pm 0.11
GF 109203X	78.7 \pm 3.9*	7.20 \pm 0.16	34.5 \pm 4.6*	6.89 \pm 0.10
OR 486	59.5 \pm 6.8	7.27 \pm 0.13	19.3 \pm 2.2	7.07 \pm 0.10
GF 109203X + OR 486	81.1 \pm 0.8*	7.23 \pm 0.13	31.1 \pm 3.4**	6.88 \pm 0.14

Data represent means \pm S.E.M. of 4 experiments, each performed in duplicate. Significantly different from control: * $P < 0.05$, ** $P < 0.01$.

Discussion

The present study has established that COMT is importantly involved in restraining the potency of isoprenaline-induced relaxation of bovine tracheal smooth muscle precontracted with methacholine, as indicated by the marked leftward shift of the concentration-response-curves of isoprenaline after inhibition of COMT (Fig 2A and 2B). Moreover, for the first time we have provided functional evidence for the possibility that activation of COMT by PKC may be involved in the reduced functional antagonism of methacholine-induced contraction by catecholamines. Thus, inhibition of COMT, PKC or the combined inhibition of COMT and PKC all enhanced the potency of isoprenaline-induced relaxation to a similar extent, strongly suggesting a common pathway for COMT and PKC, which could involve activation of COMT by PKC.

First, we assessed the effects of 10 μ M GF 109203X and 1 μ M OR 486 on methacholine-induced contraction. It was found that GF 109203X slightly reduced the potency of methacholine, with no effect on maximal contraction (Fig.1), fully in line with previous observations [13]. These findings indicate that PKC-activation by sn-1,2-diacylglycerol (DAG) is supporting contractile agonist-induced airway smooth muscle contraction mediated by G_q -coupled receptors, which has also been demonstrated by others [26-29]. As expected, OR 486 did not have any effect on methacholine-induced contraction and no additional effect on the treatment with GF 109203X either, ruling out the possibility that COMT is involved in or is interfering with methacholine-induced contraction.

Second, we demonstrated that the potency of isoprenaline on both 1 and 10 μ M methacholine-induced contraction was markedly and similarly increased in the presence of the two COMT-inhibitors OR 486 and tropolone, indicating that inactivation of isoprenaline by COMT is importantly involved (Fig. 2A and B). These findings are fully in line with other observations in pig bronchus [19] and guinea pig trachea [20], showing an increased potency of isoprenaline after inhibition of COMT using U-0521. In accordance with our results, inhibition of COMT did not effect maximal relaxation in these studies either, which of course can be explained by the fact that COMT only decreases the amount

of isoprenaline available for stimulating the β_2 -adrenoceptors present. In line with previous observations [13], with GF 109203X significant leftward shifts of the concentration-response-curve of isoprenaline, both from 1 μ M and 10 μ M methacholine-induced contraction, were observed which were remarkably similar to those obtained with OR and tropolone. These results indicate the involvement of PKC in the acute functional antagonism of contractile agonist-induced airway smooth muscle contraction by β -adrenoceptor agonists. As the gain in potency of isoprenaline was very similar after inhibition of COMT and PKC, the possibility could be envisaged that PKC and COMT may have a common pathway in reducing the β_2 -adrenergic response of isoprenaline. Therefore, we treated the smooth muscle strips with a combination of GF 109203X and OR 486. If PKC and COMT exert their effects independently, then an additive, potentiating effect on the isoprenaline-induced relaxation by the combination of the inhibitors could be expected. From both the maximal and the submaximal methacholine-induced contraction, the results showed that the combined treatment with OR 486 and GF 109203X did not give any additive effect on the relaxant potency of isoprenaline (Fig. 3A and B; Table 1), strongly suggesting a common pathway for PKC and COMT, which could involve activation of COMT by PKC-mediated phosphorylation. Interestingly, in contrast to OR 486 and tropolone, GF 109203 also increased maximal relaxation of isoprenaline, indicating an additional effect of PKC independent of COMT. To further investigate this we also studied the non-catecholamine fenoterol, which is not a substrate for COMT. As expected, OR 486 had no effect on fenoterol-induced relaxation, both from 1 and 10 μ M methacholine-induced contraction, whereas GF 109203X only increased the maximal relaxation by fenoterol. This finding confirms the idea that the observed enhanced potency of isoprenaline after inhibition of PKC is fully due to prevention of COMT-activation.

Interestingly, in patients with nocturnal asthma a temporal relationship has been observed between decreases in plasma adrenaline and decreases in peak expiratory flow rate during the early morning hours [30,31]. In contrast, the plasma histamine concentration, used as an indicator of allergic mediator secretion, rises at night in asthmatic patients and is inversely correlated with the peak flow and with the plasma adrenaline level [30]. Knowing that histamine and other mediators and neurotransmitters, released in airway inflammation, are able to activate PKC, it could be envisaged that PKC-induced activation of COMT, as found in the present study, could play a role in the decreased plasma levels of adrenaline in the early morning in patients with nocturnal asthma. Indeed, enhanced COMT-activity has been observed in children with asthma [32].

In conclusion, the results indicate that, in bovine tracheal smooth muscle preparations, activation of COMT by PKC attenuates the functional antagonism of methacholine-induced contraction by catecholamines. In addition, methacholine-induced PKC-activation is also directly involved in a reduced β -adrenergic response towards β -agonists. By reducing the β -adrenoceptor responsiveness, both PKC and COMT, as well as an interaction between the two, may therefore play a substantial role in attenuating an important, endogenous β -adrenoceptor-mediated, defence in patients with (severe) asthma.

Acknowledgements

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